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The Divergent Robo Family Protein Rig-1/Robo3 Is a Negative Regulator of Slit Responsiveness Required for Midline Crossing by Commissural Axons

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Summary

Commissural axons in vertebrates and insects are initially attracted to the nervous system midline, but once they reach this intermediate target they undergo a dramatic switch, becoming responsive to repellent Slit proteins at the midline, which expel them onto the next leg of their trajectory. We have unexpectedly implicated a divergent member of the Robo family, Rig-1 (or Robo3), in preventing premature Slit sensitivity in mammals. Expression of Rig-1 protein by commissural axons is inversely correlated with Slit sensitivity. Removal of Rig-1 results in a total failure of commissural axons to cross. Genetic and in vitro analyses indicate that Rig-1 functions to repress Slit responsiveness similarly to Commissureless (Comm) in *Drosophila*. Unlike Comm, however, Rig-1 does not produce its effect by downregulating Robo receptors on precrossing commissural axon membranes. These results identify a mechanism for regulating Slit repulsion that helps choreograph the precise switch from attraction to repulsion at a key intermediate axonal target.

Introduction

As axons grow long distances over complex terrain in the developing embryo, they make use of intermediate targets to simplify their navigation into short, manageable segments (Tessier-Lavigne and Goodman, 1996). These intermediate targets produce both attractants and repellents, which axonal growth cones must recognize in sequential order to navigate properly. Thus, after being initially attracted to their intermediate targets, growth cones must undergo a change in responsiveness to continue on their migratory route, losing responsiveness to the attractants that led them to their intermediate target and gaining responsiveness to repellents produced by that same target. This change must be tightly regulated—it must occur only after crossing, not before—so that growth cones can move on to the next stage in their trajectory only once they have passed through their intermediate target.

The ventral midline of the nervous system of both vertebrates and invertebrates has served as a model system for understanding the mechanisms by which axons interact with intermediate targets (Tessier-Lavigne and Goodman, 1996; Kaprielian et al., 2001; Yu and Bargmann, 2001). Commissural neurons, a subset of interneurons, use the ventral midline as a key intermediate target on their way to their final targets in the contralateral half of the body. In vertebrates and insects, commissural axons are initially drawn to the midline by attractant proteins, which include members of the netrin family. Upon crossing the midline and reaching the contralateral side, however, these growth cones turn longitudinally, lose responsiveness to netrins (Shirasaki et al., 1998), and become sensitive to repellents made by midline cells, which include Slit proteins (Brose et al., 1999; Kidd et al., 1999; Zou et al., 2000). This switch prevents commissural axons from recrossing the midline and allows them to move on toward their final targets.

In *Drosophila*, a single Slit protein is present and appears to account for all midline repellent activity. Commissural axons become sensitive to Slit when its receptor, Roundabout (Robo), is upregulated on the membrane of commissural growth cones upon midline crossing (Kidd et al., 1998a). Robo expression prior to reaching the midline is repressed by the regulatory protein Commissureless (Comm) (Kidd et al., 1998b), which keeps Robo in intracellular compartments away from the axonal surface (Keleman et al., 2002). Upon crossing, this repressive action of Commissureless is lost (through still unidentified mechanisms), so that Robo surface expression and, concomitantly, Slit sensitivity, are upregulated, thereby expelling commissural axons from the midline and preventing them from ever recrossing. Three mammalian homologs of *Drosophila* Slit (Slit1-3) and two homologs of Robo (Robo1, 2) were described, and their mRNAs were found to be expressed in structures analogous to those in which their homologs are expressed in *Drosophila* (midline floor plate cells for the three Slits, different subpopulations of commissural neurons for the two Robos) (Brose et al., 1999; Itoh et al., 1998; Kidd et al., 1998a; Li et al., 1999). This led to the hypothesis that this receptor-ligand system plays a similar role in vertebrate commissural axon guidance; support for this hypothesis has been obtained by our finding that spinal

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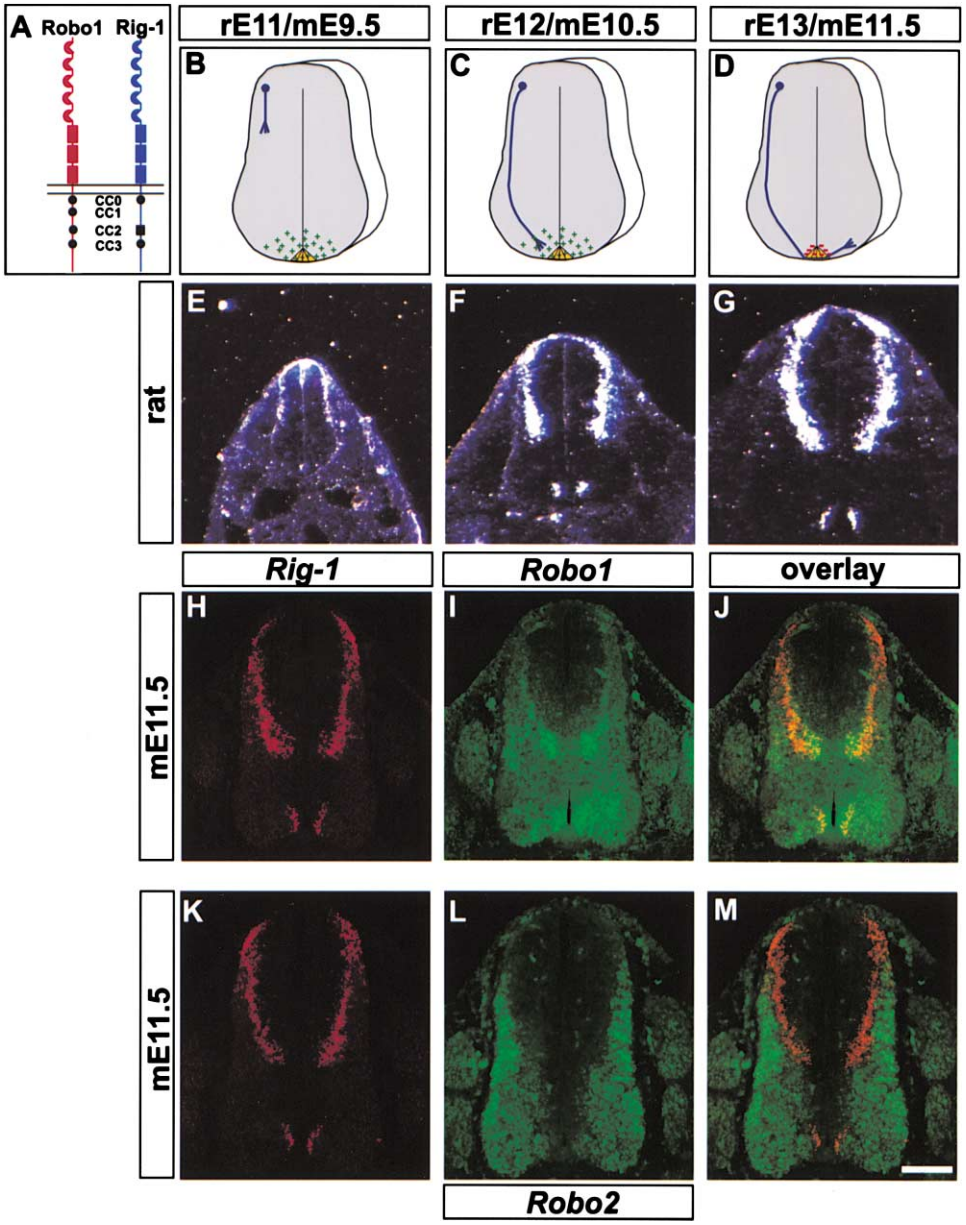

Figure 1. Expression of *mRig-1* in the Developing Rat Spinal Cord and Coexpression of *mRig-1* with *rRobo1* and *–2* in the Mouse Spinal Cord

(A) Schematic representation of the Robo1 (red) and Rig-1 (blue) domain structure. Expression of *Rig-1* at E11 (E), E12 (F), and E13 (G) in transverse sections of the rat spinal cord. *Rig-1* is not expressed in the E11 rat spinal cord (E) a time at which commissural axons have just begun their ventral migration toward the floor plate (B). By E12, when many commissural axons have reached the floor plate and a few pioneers have begun to cross (C), *Rig-1* is expressed at high levels in regions corresponding to dorsal commissural neurons as well as ventral V3 interneurons (F). *Rig-1* expression is maintained in commissural neurons at E13 in the rat (G), at which time many axons have crossed to the contralateral spinal cord and begun to grow longitudinally (D). Coexpression of *Rig-1* with *Robo1* (H–J) and *Robo2* (K–M) in transverse sections of E11.5 mouse spinal cords. As was shown for the rat spinal cord, *Rig-1* is expressed exclusively by commissural neurons in the E11.5 mouse spinal cord (H) and (K). As described previously (Kidd et al., 1998a; Brose et al., 1999), *Robo1* is expressed dorsally in the region of the commissural and association neuron cell bodies and ventrally in subpopulations of motor neurons (I). Throughout the cord, the *Rig-1* pattern of expression appears coincident with that of *Robo1* (J). As previously reported (Brose et al., 1999), *Robo2* is expressed in the motor column, in the dorsal root ganglia, and dorsolaterally along the edge of the spinal cord (L). *Robo2* expression is almost completely nonoverlapping with that of *Rig-1* (M) in both the dorsal and ventral spinal cords. Scale bar is equal to 200 μm.

commissural axons become Slit responsive upon crossing the midline (Zou et al., 2000) and fail to be efficiently expelled from the midline floor plate in *Slit1, 2, 3* triple mutant embryos (Long et al., 2004). However, no Comm homolog has yet been identified in vertebrates, raising the question of how commissural axons in vertebrates are prevented from becoming Slit responsive prior to crossing the midline.

A divergent member of the Robo subfamily, Rig-1 (also known as Robo3), was identified as a gene that

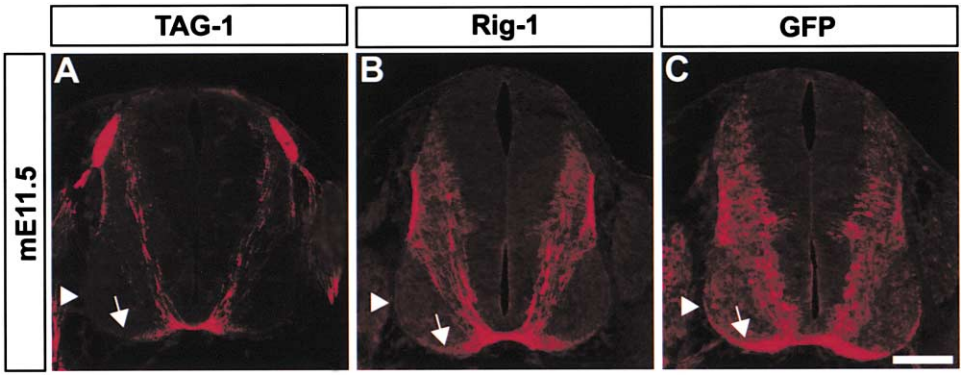

Figure 2. Rig-1 Protein Is Expressed on Commissural Axons Before and as They Cross the Floor Plate

Adjacent transverse sections of E11.5 mouse spinal cords stained with TAG1 (A), Rig-1 (B), and GFP (C). TAG1 is a marker of commissural axons that is rapidly downregulated from postcrossing axons (arrow). Similarly, Rig-1 expression although strong on commissural axons as they course ventrally toward the floor plate, also appears weaker once these axons have joined the ventral funiculus (B). This is in contrast to the GFP expression, in this case driven specifically in commissural axons by the Rig-1 promoter, which labels pre- and postcrossing commissural axons uniformly (C). Scale bar is equal to 200 μm.

is upregulated in *Retinoblastoma* (*Rb*) mutant embryos (Yuan et al., 1999). Mouse Rig-1 shares 40% amino acid identity with other vertebrate members of the Robo family, particularly in its extracellular domain, but is missing some important cytoplasmic motifs found in other Robo family members (see also Figure 1A). We found that Rig-1 is specifically expressed by commissural axons and we therefore hypothesized that Rig-1 might play a role in regulating Slit sensitivity. Unexpectedly for a Robo family member, however, Rig-1 is highly expressed before midline crossing and downregulated after crossing. Loss-of-function studies show that Rig-1 is required to allow commissural axons to enter the floor plate and cross to the contralateral side of the spinal cord. From in vitro and in vivo studies, we propose that Rig-1 normally functions to inhibit the ability of precrossing commissural axons to sense floor plate repellents of the Slit family through Robo receptors, thus allowing the axons to cross the midline.

Results

Rig-1 Binds Slit but Is Expressed on the Precrossing Portion of Commissural Axons

Since Rig-1 is a member of the Robo family, we asked whether it shares two properties of classic Robo proteins: the ability to bind Slit and expression by commissural axons. To test for Slit binding, we performed a cell overlay binding assay. As previously described, a C-terminally myc-tagged human Slit2 protein (hSlit2) binds specifically to rRobo1 but not to DCC (Brose et al., 1999). Cells expressing mRig-1 also show significant binding of hSlit2 (Supplemental Figure S1 available at http://www.cell.com/cgi/content/full/117/2/157/DC1); thus, the homology of the ectodomain of Rig-1 with those of other Slit binding Robo family members is reflected in an ability to bind Slit proteins.

To determine whether Rig-1 might contribute to commissural axon guidance, we first examined the expression of *Rig-1* mRNA in the developing rat spinal cord at embryonic stages E11-13, the time when commissural axons are projecting to the midline (Altman and Bayer, 1984). At E11, when commissural axons begin their ventral migration (Figure 1B), commissural neurons express both *DCC* and *Robo1* (Kidd et al., 1998a), but *Rig-1* expression is not detectable (Figure 1E). By E12, when commissural axons are reaching the floor plate and a few pioneers have started to cross (Figure 1C), *Rig-1* expression is upregulated specifically in commissural neurons in the dorsal spinal cord as well as V3 interneurons located on either side of the floor plate (which also project across the midline) (Figure 1F). *Rig-1* mRNA expression in commissural neurons persists through E13 (Figure 1G), a time at which many commissural axons have crossed to the contralateral side (Figure 1D).

To determine to what extent these three Robo receptors colocalize in neurons of the spinal cord, double fluorescent in situ hybridization was performed on E13 rat spinal cords. *Rig-1* expression overlaps significantly with that of *Robo1* in the dorsal spinal cord (Figures 1H–1J). In the ventral spinal cord, colocalization is also observed in the V3 interneurons. *Robo1* is also expressed in motor neurons in the ventral spinal cord whereas *Rig-1* expression appears to be confined to commissural neurons. *Robo2* was previously described to be expressed by a lateral population of dorsal interneurons as well as motor neurons at E13 in the rat spinal cord (Brose et al., 1999). Interestingly, *Robo2* expression appears largely or completely nonoverlapping with that of *Rig-1* (Figure 1K–1M).

We next examined the expression of Rig-1 protein on commissural axons, using an antibody generated against its ectodomain (Yuan et al., 1999). Unlike other Robo family proteins, we found that Rig-1 is highly expressed on the precrossing portion of the axons, as visualized by immunohistochemistry on transverse sections of E11.5 mouse spinal cord (Figure 2B) (corresponding developmentally to E13 in rat). After midline crossing, Rig-1 initially continues to be expressed by commissural axons but then gets downregulated (Figure 2B, arrowhead). The expression of Rig-1 is similar to that of TAG-1, a cell surface protein also expressed on commissural axons that gets rapidly downregulated

after midline crossing (Dodd et al., 1988). Rig-1 expression appears to persist longer than that of TAG-1 after midline crossing (at least as assessed with these particular antibodies) (Figures 2A and 2B), but it is eventually lost, as assessed by labeling adjacent sections with a GFP marker driven from the *Rig-1* locus (Figure 2C) that labels the entire length of commissural axons (see Figure 3C below).

Generation of *Rig-1* Mutant Mice

The expression of Rig-1 before crossing and its downregulation after crossing were surprising, since Rig-1 is related to Robo proteins whose expression pattern shows the opposite regulation (Long et al., 2004; see also Figure 6). We therefore sought to determine the function of Rig-1 using gene targeting in embryonic stem (ES) cells to generate mice deficient in *Rig-1*. A targeting construct was generated using a 12 kilobase fragment of a bacterial artificial chromosome containing a portion of the Rig-1 locus that includes the first exon (see Experimental Procedures). A portion of this exon encoding the start ATG and the signal sequence was replaced with a cassette containing (in order from 5′ to 3′): an internal ribosome entry site (IRES) element, a tau-GFP fusion protein, a loxP site, a PGK-1 promoter, a neomycin resistance gene, a PGK-1 polyA tail, and a second loxP site (abbreviated IRES-tauGFP-LNL and referenced in Rodriguez et al., 1999) (Supplemental Figure S2A available on *Cell* website). The IRES element was inserted to allow bicistronic expression of the tauGFP reporter from the *Rig-1* promoter.

ES cell colonies containing homologous integrants were isolated (Supplemental Figure S2B available on *Cell* website). These clones were used to generate chimeric male mice that were then mated to CD-1 or C57Bl6 females to generate germline transmissible *Rig-1*-deficient mice on either a CD-1/129Sv or a C57Bl6/129Sv genetic background. Resulting heterozygotes were crossed to generate homozygous deficient mice. Since initial experiments showed no difference in the phenotype between the two different backgrounds, most results discussed involved mutant mice on a CD-1 outbred genetic background. Homozygous deficient animals were born but lived no more than a few hours. They do not appear to suckle, since they were never observed to have milk in their bellies. However, the causes of the lethality remain unclear. To confirm that a null allele of *Rig-1* had been generated, spinal cords from *Rig-1* mutant embryos were collected, lysed, and probed by Western blotting. No Rig-1 protein was observed (Supplemental Figure S2C available on *Cell* website), confirming that the allele is likely a null.

Commissural Axons Fail to Cross the Floor Plate in the *Rig-1* Mutant

The most immediately apparent phenotype of *Rig-1*$^{-/-}$ mutant embryos was the inability of their spinal cords to stay attached at the ventral midline when dissected, indicating a thinned or fragile floor plate (data not shown). We examined commissural axons in these hemicords initially by whole-mount immunochemistry using an antibody against TAG-1. Surprisingly, the axons appear to project normally toward the floor plate,

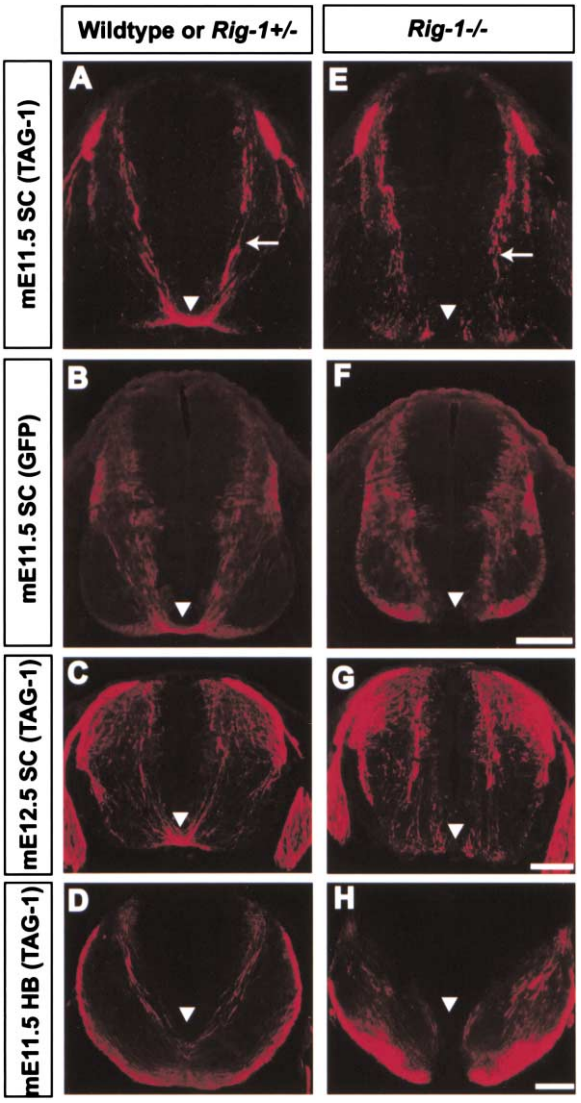

Figure 3. Lack of Commissural Axon Crossing of the Floor Plate in *Rig-1*$^{-/-}$ Mice

Visualization of commissural axons in transverse sections of wild-type or *Rig-1*$^{+/-}$ (A–D) and *Rig-1* mutant (E–H) spinal cord (A–C and E–G) and hindbrain (D and H). In wild-type and *Rig-1*$^{+/-}$ spinal cords and hindbrains, TAG1 labels commissural axons as they grow ventrally toward the floor plate (arrow in A) and as they cross to the contralateral side (arrowhead in A, C, and D). GFP driven by the Rig-1 promoter labels commissural axons along their entire lengths in *Rig-1*$^{+/-}$ embryos (B). In transverse sections of E11.5 *Rig-1* mutant spinal cords, no axons are observed crossing the floor plate as visualized by TAG1 (arrowhead in E) or GFP (arrowhead in F) although commissural axons appear to grow normally toward the floor plate in the dorsal two-thirds of the spinal cord (arrow in E). The lack of floor plate crossing is observed throughout the spinal cord and hindbrain (arrowhead in H) and persists in E12.5 spinal cords (arrowhead in G) when most commissural axons have crossed the floor plate. Scale bars are equal to 200 μm.

although upon closer inspection, they seemed to wander slightly upon reaching the ventral side of the spinal cord (Supplemental Figures S3A and S3B available on *Cell* website). To examine their trajectory in more detail, we performed TAG-1 immunostaining of transverse sec-

tions through the spinal cord of E11.5 embryos. In wild-type embryos, commissural axons project ventrally near the edge of the spinal cord until they reach the level of the developing motor column, where they turn medially to head toward the floor plate. This normal projection was observed in both wild-type and heterozygous embryos (Figures 3A, 3B, and 5A). Commissural axons in *Rig-1*$^{-/-}$ embryos, however, completely failed to cross the floor plate (Figures 3E and 3F). The initial trajectory appears normal until the vicinity of the floor plate, at which point commissural axons appear to veer away. This absence of midline crossing was also observed in sections stained with an antibody to neurofilament, which labels all axons in these sections (data not shown) as well as in sections stained with an antibody to GFP, expressed under the control of the *Rig-1* promoter (Figure 3F). Expression of the floor plate markers HNF3β and Sonic hedgehog appeared normal (data not shown) indicating that the floor plate itself develops normally in the *Rig-1* mutants.

The complete absence of ventral commissures in the *Rig-1* mutant is observed at all axial levels of the spinal cord (this study) and hindbrain (Figures 3D, 3H, and V. Marillat, C.S., V. Failli, E. Matsunaga, C. Sotelo, M.T.-L., and A. Chédotal, unpublished data) and presumably accounts for the fragility of the floor plate described above. The phenotype persists in older embryos until at least E14.5 (Figure 3G and data not shown). To assess where commissural axons go when they remain on the ipsilateral side of the spinal cord, we injected DiI in the dorsal spinal cord of E13.5 embryos visualized in an open book preparation. In wild-type embryos, by E13.5 most commissural axons have crossed the midline in a well-organized fashion and have turned sharply rostrally immediately upon exiting the floor plate (Bovolenta and Dodd, 1990). In E13.5 *Rig-1* mutant embryos, commissural axons have turned and grown longitudinally on the ipsilateral side of the floor plate. However, they are very disorganized in its vicinity. Some stall close to the floor plate and extend very large and complex growth cones. Many others, however, turn either rostrally or caudally; some actually appear to bifurcate and send projections in both directions. Most fail, however, to remain closely apposed to the floor plate, the way they normally are in wild-type embryos (Supplemental Figure S3 available on *Cell* website).

Commissural Neurons from *Rig-1* Mutant Animals Are Prematurely Slit Responsive

These studies have shown that Rig-1 is a Slit binding protein expressed on the precrossing and crossing portions of commissural axons that is required for these axons to enter the floor plate. At least two models could potentially account for these observations (Figure 4A). Model 1 postulates that Rig-1 is an attractive receptor required for the axons' response to an attractive ligand(s) (perhaps netrin-1?) that is required to enter the floor plate. Model 2 postulates that Rig-1 inhibits the axons' response to an inhibitory factor(s) secreted by the floor plate (perhaps the Slit proteins?) that prevents floor plate entry. To differentiate between these models, we first set out to characterize the responsiveness of *Rig-1* mutant commissural axons to floor plate cells in vitro, with the idea that we might be able to determine whether they have lost an attractive response or gained a repulsive response when compared to their wild-type counterparts. Commissural axons emanating from dorsal spinal cord (DSC) explants are attracted to floor plate tissue (Tessier-Lavigne et al., 1988) and COS cells secreting netrin proteins (Serafini et al., 1994) but are unresponsive to Slit2 until after they have crossed the floor plate (Zou et al., 2000). As a first step to characterizing the responsiveness of *Rig-1* mutant axons, we cultured *Rig-1* mutant DSC explants in collagen in the presence of netrin-1. The mutant axons respond normally to netrin-1 presented both as a point source (data not shown) and in the bath (Figures 4E and 4I, $p = 0.06$). Thus, Rig-1 function is not required for commissural axon responsiveness to netrin-1. However, when these explants were cocultured with floor plate tissue derived from wild-type embryos, which is a potent source of netrin-1 (Serafini et al., 1996), axons failed to grow out of the explant (Figures 4C and 4H, $p < 0.001$). The fact that commissural axons from mutant explants grow out into collagen in response to recombinant netrin-1 but not floor plate-derived netrin suggests that something else made by floor plate cells is antagonizing netrin's action. To test whether Slit proteins secreted by floor plate might be antagonizing netrin's action, we cocultured *Rig-1* mutant explants with floor plate in the presence of an antagonist of Slit function provided by the ectodomain of Robo2 (fused to the Fc portion of human IgG). Remarkably, bath-applied Robo2-Fc ectodomain rescued outgrowth of axons from *Rig-1* mutant explants in the presence of floor plate (Figures 4D and 4H); the amount of outgrowth approached that seen with wild-type explants in the presence of floor plate (as assessed by total axon bundle length per explant; $p < 0.001$).

These results suggested that Slit proteins derived from the floor plate were antagonizing the action of floor plate-derived netrin on commissural axons from *Rig-1* mutant DSC explants. To test more directly whether these axons are prematurely responsive to Slit proteins, we cultured DSC explants with netrin-1 to elicit commissural axon outgrowth, and presented the axons with COS cells secreting the N-terminal cleavage product of Slit2, a potent repellent of a variety of different axonal populations (e.g., Nguyen Ba-Charvet et al., 1999). Axons emanating from wild-type DSC explants are not repelled by Slit2-expressing COS cells unless they have already crossed the floor plate (Zou et al., 2000; Figures 4E and 4I). In contrast, the growth of axons from *Rig-1* mutant DSC explants, which have not encountered the floor plate, is strongly inhibited by Slit2N-expressing cells (Figures 4G and 4I; $p < 0.001$).

These in vitro results support a role for Rig-1 as an inhibitor of Slit signaling in commissural axons prior to crossing the floor plate rather than implicating Rig-1 in mediating the response to a floor plate attractant, and thus strongly support model 2 (Figure 4A). Interestingly, although axons from *Rig-1*$^{-/-}$ DSC explants were prematurely responsive to Slit2N, they were not inhibited by Sema3F (Figure 4F), a distinct repellent known to inhibit postcrossing commissural axons (Zou et al., 2000). The fact that loss of Rig-1 does not result in loss of netrin responsiveness or in premature responsiveness to Sema3F shows that loss of Rig-1 specifically causes

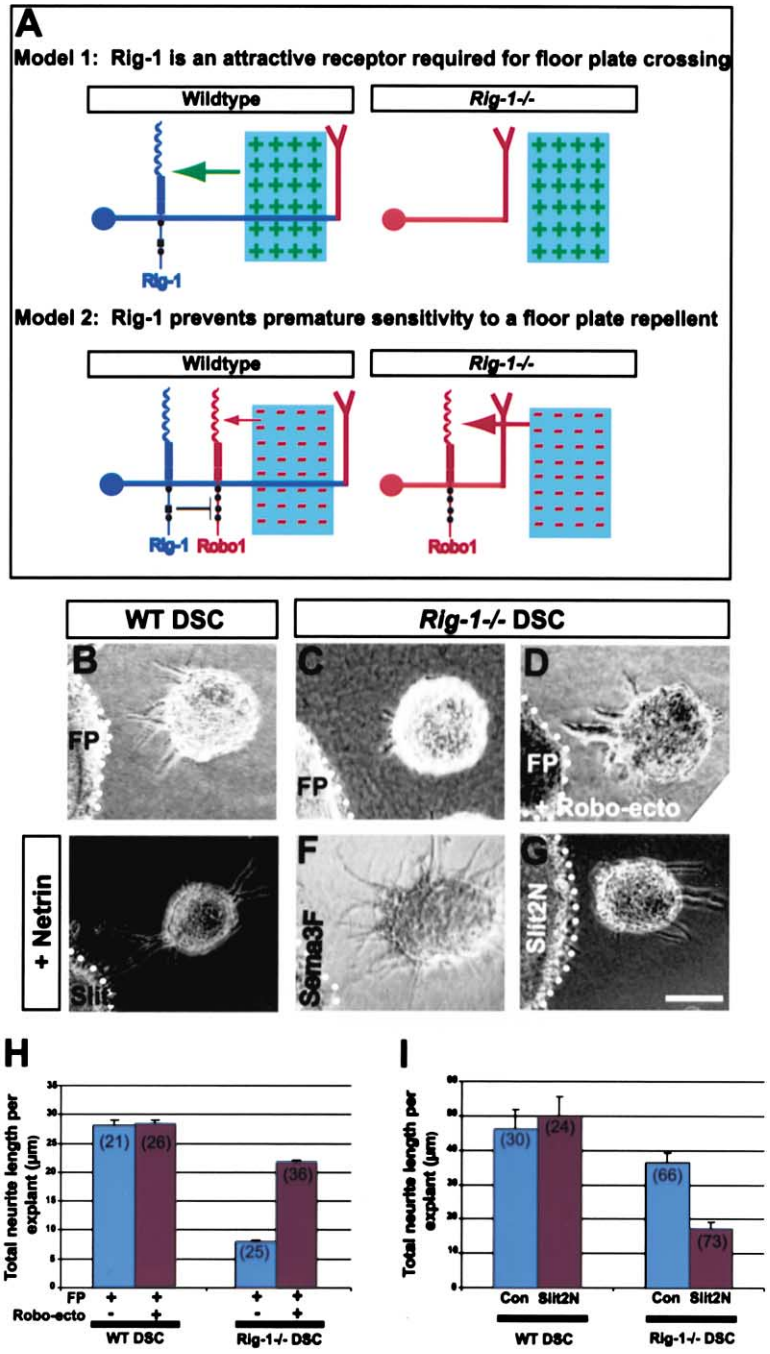

Figure 4. *Rig-1* Mutant Commissural Axons Are Repelled by Slit Protein in the Floor Plate Prior to Crossing

(A) Two models could account for the lack of midline crossing observed in the *Rig-1* mutant spinal cords. Model 1 postulates that Rig-1 may function as a receptor that responds to an attractant in the floor plate. In the absence of Rig-1, commissural axons fail to respond to this floor plate attractant and thus fail to cross to the contralateral side of the spinal cord. Model 2 postulates that Rig-1 inhibits the repulsive effects of a ligand expressed by the floor plate. For example, Rig-1 may prevent a repellent such as Slit from activating Robo1 on commissural axons prior to crossing. However, once commissural axons have crossed the floor plate, Rig-1 is downregulated and Slits in the floor plate become able to repel commissural axons. In the absence of Rig-1, Slits are able to activate Robo1 on commissural axons at any time and thus prevent commissural axons from ever crossing the floor plate.

In vitro collagen cultures of DSC explants were used to distinguish between these two models (B–G). Commissural outgrowth was elicited from DSC explants by coculturing with floor plate (FP) (B–D) or including 125 ng/ml purified Netrin-1 in the bath (E–G). Commissural axons grow out of DSC explants from WT spinal cords in response to FP (B). However, *Rig-1*$^{-/-}$ commissural axons fail to grow out significantly when cocultured with WT FP (C). The lack of commissural axon outgrowth from *Rig-1*$^{-/-}$ DSC in response to FP can be rescued by the inclusion of purified Robo2-ectodomain fused to Fc in the culture medium (D). Commissural axons grow out into collagen from DSC explants in response to the presence of Netrin-1 in the culture medium. When confronted with COS cells expressing the N-terminal fragment of hSlit2 (Slit2-N), Netrin-responsive commissural axons from WT DSC explants grow normally and are not repelled (E). However, commissural axons from *Rig-1*$^{-/-}$ DSC explants are strongly repelled by Slit2-N (G) but unaffected by another repellent found in the floor plate, Sema3F (F). The results of these in vitro experiments are quantified in (H) and (I). Explants from at least four different embryos were analyzed for each condition described. The number of explants quantified is indicated in parentheses in (H) and (I). Scale bar is equal to 100 μm.

the axons to become prematurely Slit responsive, rather than generally converting them to a postcrossing state.

Removal of *Slits* in a *Rig-1* Mutant Background Leads to Partial Rescue of the Crossing Phenotype

Taken together, these results strongly suggested that failure of axons to cross the midline in *Rig-1* mutant spinal cords results from the axons being prematurely responsive to midline Slit proteins, which block their entry into the midline. To test this hypothesis, we genetically removed individual Slits from the floor plate in the *Rig-1* mutant background by crossing *Slit1* and *Slit2* mutants with the *Rig-1* mutant. At the level of commissural axon crossing of the floor plate, neither *Slit1*$^{-/-}$, *Slit2*$^{-/-}$, nor *Slit1*$^{-/-}$;*Slit2*$^{-/-}$ mutants have any obvious phenotype (Plump et al., 2002; Long et al., 2004), (although removal of all three Slits causes stalling at the floor plate; Long et al., 2004). Since the loss of ventral commissures in the *Rig-1* mutant is so complete, we reasoned that any decrease in Slit-dependent repulsion from floor plate might be translated into some recovery of crossing. *Slit1*$^{-/-}$; *Rig-1*$^{-/-}$ embryos showed no midline crossing by commissural axons and looked identical to the *Rig-1*$^{-/-}$ littermates (Figures 5A and 5B). On the other hand, *Slit2*$^{-/-}$; *Rig-1*$^{-/-}$ as well as *Slit1*$^{-/-}$; *Slit2*$^{-/-}$; *Rig-1*$^{-/-}$ triple mutants embryos showed recovery of

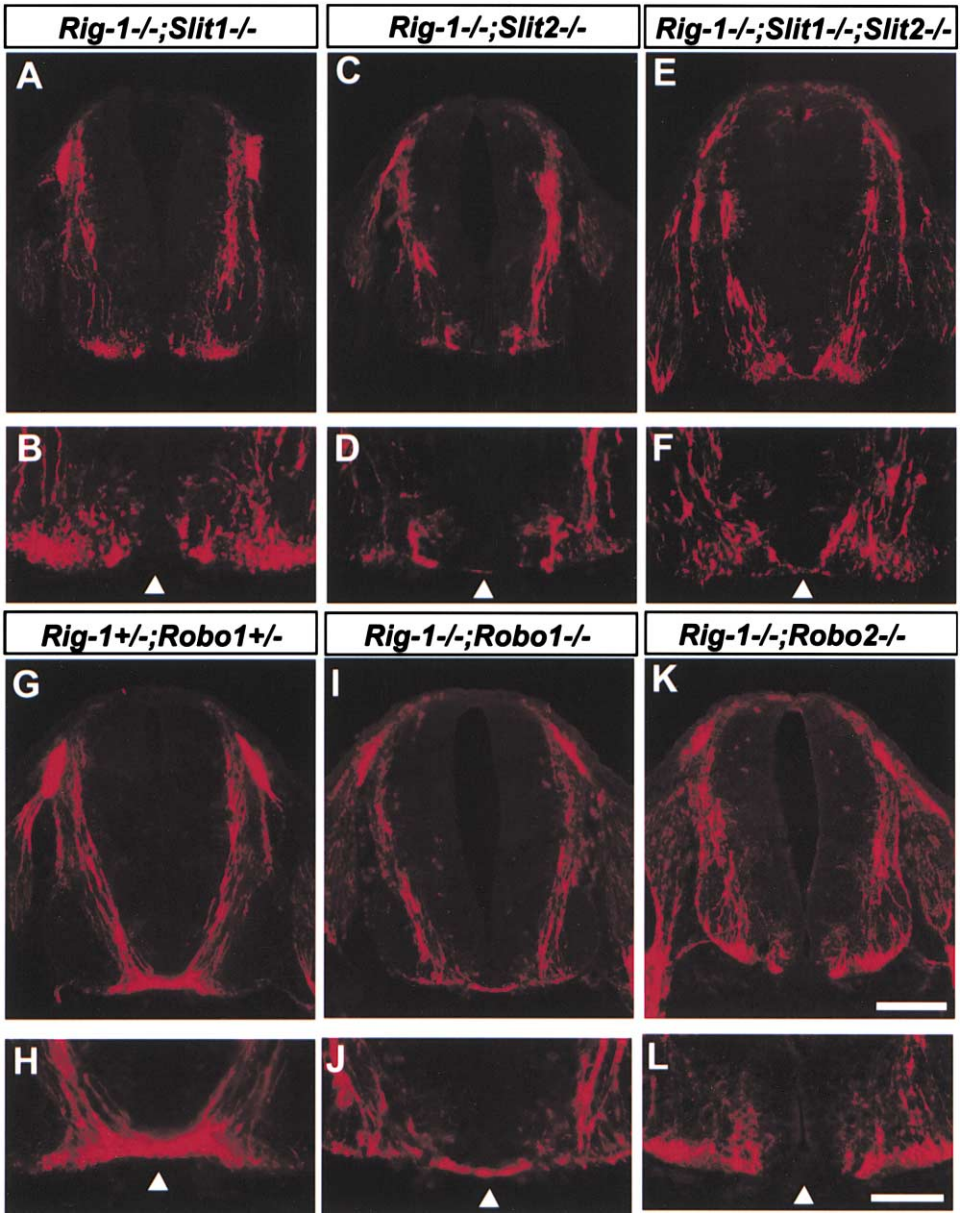

Figure 5. Removal of Slits or of Robo1, but Not Robo2 Can Rescue Midline Crossing in the *Rig-1* Mutant Spinal Cord

In transverse sections of wild-type or *Rig-1*$^{+/-}$;*Robo1*$^{+/-}$ spinal cord, TAG1 labels commissural axons as they grow ventrally toward the floor plate and as they cross the floor plate in a thick bundle (G and H). In *Rig-1*$^{-/-}$, *Rig-1*$^{-/-}$;*Slit1*$^{-/-}$ (A and B) or *Rig1*$^{-/-}$;*Robo2*$^{-/-}$ (K and L) double mutants, no TAG1-positive commissural axons are observed crossing the floor plate. Some rescue of midline crossing is observed in *Rig-1*$^{-/-}$;*Slit2*$^{-/-}$ double mutants (C and D) as shown by the small amount of TAG1 immunoreactivity observed in about half of the sections in the floor plate of those double mutants (arrowhead in D = 3 embryos). Rescue of midline crossing was also observed in *Rig-1*$^{-/-}$;*Slit1*$^{-/-}$;*Slit2*$^{-/-}$ triple mutant spinal cords (E and F; n = 2 embryos). In this case, crossing axons were observed in every section. Significant rescue of midline crossing was observed in Rig-1$^{-/-}$;Robo1$^{-/-}$ embryos (n = 4), with many axons seen crossing in every section (I and J). However, the thickness of the commissural bundle in the floor plate was still not back to wild-type levels (G and H). On the other hand, Rig-1$^{-/-}$;Robo2$^{-/-}$ double mutants (n = 4) look indistinguishable from Rig-1$^{-/-}$ embryos (K and L).
Scale bar is equal to 200 μm (A, C, E, G, I, and K). Scale bar is equal to 100 μm (B, D, F, H, J, and L).

midline crossing by commissural axons at all axial levels of the spinal cord and the hindbrain, although a majority still failed to cross (Figures 5C–5F). The expressivity of the recovery is greater in the triple than the double mutant (see Figure 5 legend). Thus, loss of Slit function can rescue at least partly the *Rig-1* mutant phenotype, consistent with the model that loss of Rig-1 in vivo results in absence of midline crossing because commissural axons are prematurely Slit responsive.

Loss of *Robo1* but Not *Robo2* Partially Suppresses the *Rig-1* Mutant Phenotype

Robo1 and Robo2 are the presumed Slit receptors mediating repulsive actions of Slits on different populations

of commissural axons. In our companion paper, we report that loss of either *Robo1* or *Robo2* alone results in only a slight midline crossing phenotype (Long et al., 2004), presumably due to redundancy between the two Robos, and/or to the functioning of other repulsive guidance systems at the midline, including the Semaphorin/Neuropilin-signaling system (Zou et al., 2000). Whether or not other signaling systems are normally involved in repelling axons out of the midline, however, we predict that removal of Robo function should suppress the phenotype of *Rig-1* mutant embryos, provided the Robo proteins contribute to signaling midline repulsion by Slit proteins in vivo. To test for such suppression, we examined the effects of mutating either Robo1 or Robo2 in the *Rig-1*$^{-/-}$ background. A dramatic effect of removing *Robo1* was observed: significant midline crossing was seen in E11.5 *Rig-1*$^{-/-}$*; Robo1*$^{-/-}$ double-mutant embryos as visualized by TAG-1 (Figures 5I and 5J) and neurofilament (data not shown) staining. This partial suppression of the *Rig-1*$^{-/-}$ mutant phenotype appears specific to Robo1 as it was not observed in *Rig-1*$^{-/-}$*; Robo2*$^{-/-}$ double mutants (Figures 5K and 5L). This is presumably related to the fact that *Rig-1* expressing neurons in the spinal cord primarily express *Robo1*, not *Robo2* (Figure 1).

Robo1 and 2 Proteins Are Localized Primarily to the Longitudinal Portion of Axons in Both Wild-Type and *Rig-1*$^{-/-}$ Embryos

The *Rig-1*$^{-/-}$ phenotype in the spinal cord is reminiscent of the *Drosophila commissureless* (*comm*) phenotype, in which commissural axons also fail to cross the CNS midline (Kidd et al., 1998b). Commissureless (Comm) inhibits Robo signaling by preventing Robo from being targeted to the plasma membrane before and during midline crossing (Keleman et al., 2002). To test whether Rig-1 functions in a similar manner, we used antibodies against the ectodomains of Robo1 and Robo2 to determine their localization in commissural axons in wild-type and *Rig-1*$^{-/-}$ embryos. Robo1 and Robo2 are localized primarily to the postcrossing portion of the axon, although low levels are observed precrossing (Long et al., 2004) (see also Figures 6B and 6C). Unexpectedly, Robo1 and Robo2 immunoreactivity appears unchanged in *Rig-1*$^{-/-}$ embryos, i.e., low levels are observed prior to reaching the midline, and dramatic upregulation of expression is observed once the axons are coursing in the ventral funiculus, indicating that both receptors are confined to the longitudinal portion of commissural axons even though these axons have not crossed the floor plate (Figures 6E and 6F). This observation is distinct from what is observed in *Drosophila comm* mutants, in which Robo localization is abnormally expressed on precrossing commissural axons. It also implies that the low levels of Robo protein expression observed prior to crossing must be sufficient to mediate Slit responsiveness in these axons.

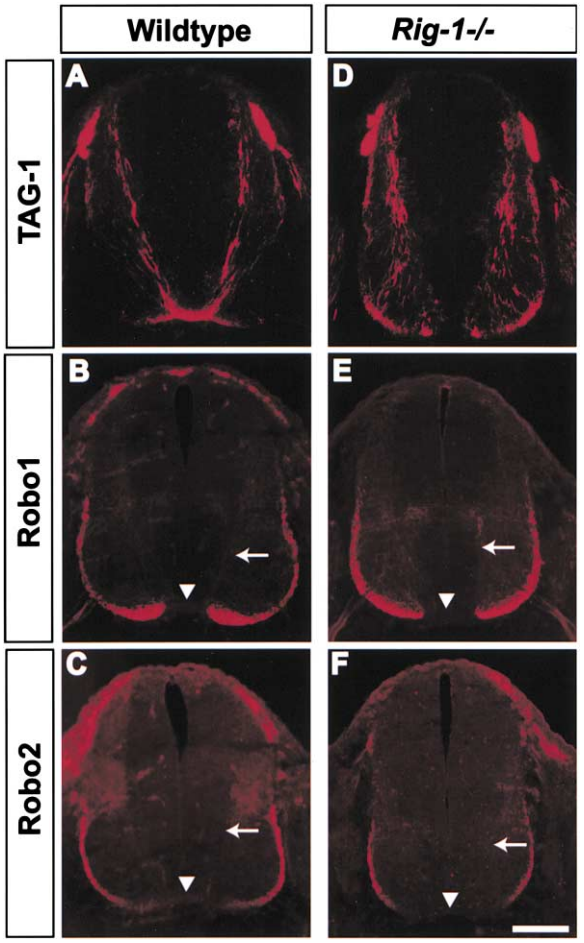

Figure 6. Robo1 and Robo2 Localization Is Unchanged in *Rig-1* Mutant Spinal Cords

Commissural axons are labeled with TAG1 until they exit the floor plate in wild-type spinal cords (A) or as they course ventrally in the *Rig-1*$^{-/-}$ spinal cord (D). Immunohistochemistry against Robo1 protein labels precrossing (arrow in B) and crossing commissural axons at low levels (arrowhead in B). Once commissural axons enter the ventral funiculus, Robo1 levels are dramatically upregulated (B). Surprisingly, Robo1 localization appears unaltered in *Rig-1* mutants: Robo1 levels are kept low as commissural axons grow ventrally toward the floor plate (arrow in E) and become high after they enter the ventral funiculus despite the lack of crossing of the floor plate (arrowhead in E). Similarly, Robo2 is primarily expressed in a subset of axons growing in the ventral funiculus with low levels also observed in precrossing and crossing commissural axons (C). Robo2 localization is unchanged in *Rig-1*$^{-/-}$ embryos (F). Scale bar is equal to 200 μm.

Discussion

As axons grow toward their final targets, they interact in a highly regulated fashion with a series of intermediate targets that guide them using both attractants and repellents. Commissural axons in the spinal cord are initially attracted to the floor plate, but upon crossing it, they lose responsiveness to floor plate attractants (Shirasaki et al., 1998) and become responsive to floor plate repellents of the Slit and Semaphorin families (Zou et al., 2000). To be effective, this change in responsiveness to floor plate-derived guidance cues must be tightly linked to crossing of that intermediate target. Our results support a model in which Rig-1/Robo3, a member of the roundabout receptor family, keeps commissural axons from sensing ligands of the Slit family through their cognate receptor Robo1 as they grow toward the floor plate,

allowing them to enter and cross to the contralateral side, where downregulation of Rig-1 protein expression helps them sense the floor plate as a repulsive environment, thus preventing them from recrossing the midline.

Rig-1 Is Required for Commissural Axons to Cross the Floor Plate

Our finding that *Rig-1* mRNA is expressed highly and selectively in commissural neurons initially suggested that Rig-1, as a member of the Robo family, might play a role in preventing midline recrossing, similar to the role played by Robo in *Drosophila*. We were thus surprised by an unexpected phenotype in *Rig-1* mutants: a complete failure to enter the ventral midline region, reflected in the lack of ventral commissures throughout the spinal cord and hindbrain. This result indicated that Rig-1 plays a role in guiding commissural axons prior to crossing the midline, consistent with the pattern of expression of the Rig-1 protein: high prior to crossing and low postcrossing. This expression pattern stands in contrast to those of Robo1 and Robo2, which are observed primarily postcrossing (although both are also observed at low levels in precrossing and crossing commissural axons).

The *Rig-1* mutant phenotype superficially shares some features with the *Netrin-1* and *Dcc* mutant phenotypes, in which ventral commissures are also severely reduced in the spinal cord. There are, however, profound differences. In the *Netrin-1* or *Dcc* mutants, commissural axons are impaired in their ability to grow ventrally toward the floor plate, and few reach the midline (Fazeli et al., 1997; Serafini et al., 1996). Thus, in those mutants the reduced ventral commissure reflects a failure to reach the midline. In *Rig-1* mutant embryos, in contrast, commissural axons apparently grow normally, virtually all the way to the floor plate, deviating from their normal ventral migration only as they get close to it (Figure 3E). Furthermore, whereas in both *Netrin-1* and *Dcc* mutants a few commissural axons are observed crossing to the contralateral side through the floor plate, no axon was ever observed in the floor plate of *Rig-1* mutant spinal cords at any of the ages analyzed (Figure 3 and data not shown). These phenotypic distinctions suggest that Rig-1 is unlikely to be involved in sensing the attractant Netrin-1, an interpretation further supported by our in vitro experiments that show normal outgrowth in response to Netrin-1 of commissural axons from *Rig-1*$^{-/-}$ dorsal spinal cord explants (Figures 4F and 4I).

Rig-1 Inhibits the Responsiveness of Commissural Axons to Slit Repellents

Thus, rather than being involved in commissural axon guidance to the midline, Rig-1 instead appears to be required specifically for midline crossing. Our results strongly support a role for Rig-1 in preventing commissural axons from becoming prematurely responsive to Slit repellents. In vitro, we found that commissural axons from *Rig-1* mutant dorsal spinal cord explants are repelled by COS cells secreting Slit2N, to which their wild-type counterparts are insensitive. This premature Slit sensitivity provides an explanation for the observation that *Rig-1* mutant commissural axons fail to grow out of dorsal spinal cord explants in response to floor plate tissue, since inhibition by floor plate-derived Slit could override the outgrowth-stimulating effect of netrin. This interpretation is supported by the finding that the outgrowth is restored when a soluble Robo-ectodomain is added to the culture medium, presumably blocking the effect of the Slit proteins. The in vivo counterpart of this in vitro experiment was to remove *Slit1* and *Slit2* in the *Rig-1* mutant background, which led to a partial rescue of commissural axon crossing, again consistent with the possibility that commissural axons fail to cross the midline in *Rig-1* mutants because of premature Slit sensitivity.

It could be argued that the partial suppression of the *Rig-1* midline-crossing defect by removal of *Slit-1* and *Slit-2* might be due simply to the floor plate being a more generally attractive environment in the absence of Slits. Two lines of evidence argue against this alternative interpretation, however. First, no defect in commissural axon guidance at the floor plate has been detected in the *Slit1;Slit2* double-mutant embryos despite thorough analysis (Plump et al., 2002). In fact, recent results have shown that only when all three Slit proteins expressed by the floor plate are removed (in *Slit1;Slit2;Slit3* triple mutants) does a commissural axon guidance defect become apparent (Long et al., 2004). These observations imply that the balance of attractants and repellents in the floor plate is not significantly altered by removal of Slit1 and Slit2. Secondly, removal of Neuropilin-2 in the *Rig-1* mutant background fails to rescue crossing (Supplemental Figure S3 available on *Cell* website). Commissural axons in *Neuropilin-2* mutants stall out in the floor plate at high frequency, suggesting that upon crossing they sense the floor plate as a less repulsive environment than in wild-type animals (Zou et al., 2000). The fact that no rescue of the *Rig-1*$^{-/-}$ crossing phenotype is observed in the *Neuropilin-2;Rig-1* double mutants indicates that sensing fewer repellents in the floor plate per se is not sufficient to allow *Rig-1* mutant commissural axons to cross the floor plate, and that instead it is Slit repulsion specifically that must be lessened for the rescue of midline crossing. This conclusion is further supported by the observation that commissural axons from *Rig-1* mutant animals become prematurely responsive in vitro to Slit2N but not to Sema3F, a Neuropilin-2 ligand.

Thus, taken together, both our in vitro and in vivo results support a model in which *Rig-1* mutant commissural axons fail to cross the midline in vivo specifically because they are prematurely responsive to Slit repellents (Model 2 in Figure 4). It should be noted, however, that it remains formally possible that in addition to this inhibitory role, Rig-1 may also recognize an attractant in the floor plate that, unlike Netrin-1, is not responsible for drawing commissural axons ventrally but is important for crossing the floor plate (Model 1 in Figure 4). This issue may only be resolved if a putative attractive function of Rig-1 can be separated from its role in preventing repulsion, for example through structure-function studies. It should be stressed, however, that this remains only a formal possibility, and that an inhibitory effect of Rig-1 on Slit responsiveness by commissural axons is sufficient by itself to explain all of the observed *Rig-1* mutant phenotypes as well as our in vitro results.

To confirm the role of Rig-1 as an inhibitor of the Slit

response, we assessed the effect of removing the receptors for Slit on the *Rig-1* mutant phenotype. Interestingly, whereas removal of Robo1 leads to rescue of commissural axon crossing in the *Rig-1*$^{-/-}$ background, removal of Robo2 has no effect on the *Rig-1*$^{-/-}$ phenotype (Figure 5). The lack of rescue in the *Robo2*$^{-/-}$;Rig-1 double mutant is presumably explained by the expression of *Robo2* mRNA in the spinal cord, which is almost entirely nonoverlapping with that of *Rig-1* (Figure 1M). Therefore, the complete absence of both Robo1-positive and Robo2-positive fibers in the ventral commissures of the Rig-1 mutant spinal cord indicates that Robo2-expressing axons may be dependent on Rig-1 in a cell-nonautonomous manner to cross the midline. For example, Robo1-expressing axons may pioneer floor plate crossing and Robo2-expressing axons may fasciculate onto the Robo1-positive pioneers. Consistent with this hypothesis, commissural axons are observed stalled in the floor plate of *Robo1* mutants at early time points, a phenotype that fits with a role for Robo1 in sensing Slits in the floor plate. On the other hand, Robo2 mutant commissural axons appear to cross the floor plate normally (Long et al., 2004). Together these observations lead us to conclude that in the absence of Rig-1, Robo1-positive commissural axons fail to enter the floor plate because they are repelled by Slit proteins, and the Robo2-positive fibers then fasciculate with the misguided Robo1-positive axons, resulting in a failure to cross as well.

The lack of complete rescue of the *Rig-1* mutant phenotype by removal of Robo1 has several possible explanations. First, as already suggested by the subtle commissural axon guidance phenotype observed in the *Robo1* single mutants (Long et al., 2004), Robo1 might not be the only receptor for Slit proteins on Rig-1 expressing commissural axons. Second, it remains possible that Rig-1 blocks premature responsiveness to yet other repellents in the floor plate beside Slits, although as argued above, such repellents would presumably not be ligands for Neuropilin-2 like Sema3B and Sema3F. Finally, there could be a small amount of residual Robo1 protein in the *Robo1* knockout, because of a small amount of splicing over the gene trap insertion, that results in presence of a small amount of wild-type *Robo1* mRNA in these animals (Long et al., 2004). Whatever the explanation, the significant rescue of midline crossing observed in *Robo1;Rig-1* double mutants strongly implies that Rig-1 inhibits Slit signaling through Robo1 in commissural axons.

Crossing the Midline in Vertebrates and *Drosophila*

The lack of a ventral commissure in the spinal cord and hindbrain of *Rig-1* mutant mice is analogous to the complete absence of commissures in the CNS of *comm* mutants in *Drosophila* (Tear et al., 1996). Like Rig-1, Comm has been shown to inhibit Slit responsiveness in commissural axons prior to crossing (Kidd et al., 1999; Keleman et al., 2002). As commissural axons grow toward the midline, Comm interacts with *Drosophila* Robo1 (DRobo1) and prevents its localization to the axonal membrane. Once commissural axons have crossed the midline, Comm's inhibition of DRobo1 is relieved. In *comm* mutant embryos, DRobo1 is mislocalized to the axonal membrane as commissural axons approach the midline causing commissural axons to become sensitive to Slit prior to crossing. In mouse, as in *Drosophila*, Robo1 and Robo2 are primarily localized to the postcrossing portion of the axon (Figures 6B and 6C). However, in *Rig-1* mutants, Robo1 and Robo2 protein expression is not upregulated in commissural axons as they grow ventrally toward the floor plate. Rather, both proteins still appear to be confined to the "postturning" commissural axons as they grow longitudinally—but in this case in the ipsi—rather than the contralateral spinal cord (Figures 6E and 6F). We conclude, therefore, that Rig-1 inhibits Slit responsiveness via a different mechanism than Comm. This conclusion is corroborated by biochemical experiments in which we found that under conditions where Comm and DRobo1 interact in transfected COS cells (Keleman et al., 2002), Rig-1 and Robo1 do not show any biochemical interaction (data not shown). Similarly, whereas Comm has been shown to relocalize DRobo1 from the surface of transfected COS cells to intracellular compartments (Keleman et al., 2002), Rig-1 has no such effect on Robo1 in vitro (data not shown). Thus, Rig1 appears to produce its effect not by affecting Robo protein expression, but rather by preventing Slit signaling via the small amount of Robo protein that is present on the axons precrossing; a corollary of this is that the small amount of Robo that is present must be enough to mediate a sufficient response to midline Slit proteins to prevent crossing in the absence of Rig-1.

Does this mean that flies and vertebrates have hit on completely different solutions to the problem of preventing premature Slit responsiveness? Not necessarily. First, in vertebrates, like flies, Robo protein expression is dramatically upregulated after midline crossing. It is possible that this regulation of protein localization uses a similar mechanism to that operating in flies, perhaps using a still to be discovered Comm-like protein. Second, although studies in *Drosophila* have focused on the role of Comm in regulating Robo protein expression, there is nonetheless some Robo expressed precrossing, and it is tempting to speculate that there must be some specific second mechanism to prevent that Robo from signaling sufficient repulsion to prevent crossing. Thus, flies and vertebrates might both have two mechanisms: one to regulate Robo protein expression (involving Comm in flies and an unknown mechanism in vertebrates) and one to silence low level Robo protein precrossing (involving Rig-1 in vertebrates and an unknown mechanism in flies). A second mechanism in flies need not require a second molecule: Comm itself could, in principle, silence Robo precrossing independent of its effect on protein localization.

How does Rig1 inhibit the response to Slit through Robo1? Since we have shown that Rig-1 can directly bind Slit proteins, Rig-1 might behave as an endogenous dominant-negative Robo receptor and bind Slit unproductively, sequestering it away from Robo1. Although the lack of a phenotype in Rig-1 heterozygous animals tends to argue against this hypothesis, it is possible that Rig-1 is present in such vast excess over Robo1 on precrossing commissural axons that a reduction of its levels by half would not affect its ability to inhibit Robo1. Alternatively, the divergence of the Rig-1 cytoplasmic

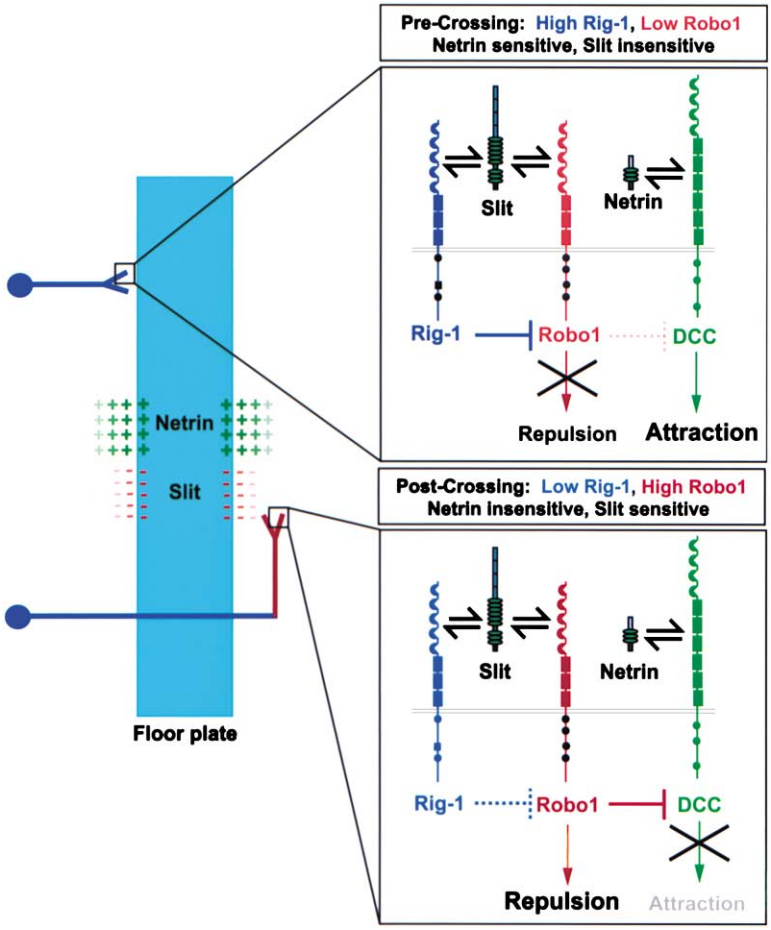

Figure 7. Model for the Switch in Ligand Responsiveness as Commissural Axons Cross the Floor Plate

Diagram of molecular interactions on precrossing commissural axon growth cones. Precrossing growth cones are attracted to the floor plate primarily through the effect of Netrin-1 on its receptor DCC (as well as by attraction by Shh [not illustrated]). Slit1-3, repulsive ligands also expressed by the floor plate, are recognized by Robo1 on commissural axon growth cones, but the presence of Rig-1/Robo3 on the growth cone membrane inhibits Robo1 from eliciting a repulsive response to these ligands. After crossing the floor plate, the inhibition of Slit responsiveness is relieved due to the absence of Rig-1 on postcrossing commissural axons and the concomitant upregulation of Robo protein expression. This coincides with a loss of responsiveness to Netrin-1, presumably due to the interaction between Robo1 and DCC. Upregulation of responsiveness to the repellent Sema3B also occurs (not illustrated). The downregulation of responsiveness to attractants and upregulation of responsiveness to repellents expels the axons out of the midline, onto the next leg of their trajectory.

domain compared to other Robo family members suggests that Rig-1 may signal differently than Robo1 and may interfere with Robo1 downstream signals.

Understanding the Midline Switch in Commissural Axon Guidance

How does Rig1 fit in more broadly with the high fidelity switch from attraction to repulsion that occurs at the midline? As commissural axons grow ventrally toward the floor plate, they are attracted by Netrin-1 via the netrin receptor DCC (Keino-Masu et al., 1996; Serafini et al., 1996). In vitro, Robo1 can silence netrin responsiveness through its direct interaction with DCC in the presence of Slit (Stein and Tessier-Lavigne, 2001). The lack of inhibition of DCC in the precrossing commissural axon may be mediated by both the low levels of Robo1 present in commissural axons prior to crossing the floor plate and by the inhibition of Robo1 by Rig-1 in precrossing axons. Both phenomena together would ensure that commissural axons initially sense the floor plate as an overwhelmingly attractive environment (Figure 7). Once they have interacted with the floor plate and entered the contralateral side of the spinal cord, a cascade of events takes place that ultimately leads to a rapid change in direction from the dorsal-ventral axis to the anterior-posterior axis. The downregulation of Rig-1 and coincident upregulation of Robo1 would work together to ensure that commissural axons move past their intermediate target and stay on course in the contralateral ventral funiculus. Both these events may be required to finely tune the switch at the midline. Rig-1 is observed at low levels on the postcrossing portion of commissural axons but is by no means completely turned off immediately upon entering the contralateral spinal cord (Figure 2). Thus, the upregulation of Robo1 may be a means to overwhelm the Rig-1 inhibition prior to it being completely downregulated. Alternatively, Rig-1 function may be inhibited through other means besides protein downregulation so that tight control over the upregulation of the Slit response is achieved. Rig-1 downregulation and the subsequent disinhibition of Robo1 not only permits upregulation of Slit responsiveness, but may also make Robo1 available to bind DCC and thus silence Netrin-1 responsiveness. Together, these two events would convert commissural axons from sensing the floor plate as an attractive environment to sensing it as a repulsive environment (Figure 7), repelling them out of the midline and allowing them to move onto the next leg of their trajectory.

Experimental Procedures

Generation of *Rig-1*-Deficient Mice

Genomic DNA containing portions of the Rig-1 gene was isolated by screening a BAC library (Incyte Genomics) with a Rig-1 specific cDNA probe. BAC DNA was then used to generate the targeting vectors shown in Supplemental Figure S2A (available on *Cell* web-

site) using standard recombinant DNA techniques. Southern blot and Western blot analyses were performed using standard techniques. To identify targeting events, genomic DNA was digested with PstI and hybridized with a DNA probe external to the targeting vector as noted in the targeting figures. ES cell culture and generation of mice was carried out as previously described (Mombaerts et al., 1996). For genotyping, a PCR-based screen was developed: wild-type allele-forward primer 5′-TACCAGCTACTTCCAGAGAG-3′; reverse primer 5′-CCAACATCGAGTGGTACAAG-3′; mutant allele forward-primer 5′-GATCTCTCGTGGGATCATTG-3′; reverse primer 5′-TACCAGCTACTTCCAGAGAG-3′. PCR was carried out using the same protocol previously described (Plump et al., 2002).

Commissural Axon Outgrowth Assays

Explants of E11.5 mouse dorsal spinal cord were isolated and cultured as described for rat explants, except that they were cultured in 45% OptiMEM-1 (GIBCO BRL), 50% F12 (GIBCO BRL), 5% heat-inactivated horse serum, 40 mM glucose, 2 mM GlutaMAX I (GIBCO BRL), 100 μg/ml streptomycin sulfate, and 100 U/ml penicillin G. Dorsal spinal cord explants were cocultured with E11.5 mouse floor plate, or COS cell aggregates transfected with a control, Slit2-N, or Sema3F-expression plasmid. When indicated, outgrowth of commissural axons was elicited by adding 125 ng/ml of purified Netrin-1 to the culture media. Explants were fixed and analyzed after 18–20 hr.

COS Cell Aggregates

For the production of transfected COS cell aggregates, COS cells were plated in a 6-well dish and transfected 16 hr later with 1 μg of DNA (pSecTagB, pSecTag-Slit2N, pSecTag-Sema3F) using 3 μl of FuGene 6, according to the manufacturer's protocol. 24 hr after transfection, cell layers were trypsinized, washed with DME-10% FBS, and resuspended in 150 μl DME-10% FBS. Drops of the cell suspension (20 μl) were placed onto the lids of 12-well dishes, which were inverted over dishes containing DME. These hanging drop cultures were incubated for 6–12 hr and aggregates were trimmed with tungsten needles.

Immunohistochemistry

Embryos were fixed overnight at 4°C in 4% paraformaldehyde (PFA)/phosphate-buffered saline (PBS), washed with PBS, incubated in 30% sucrose/PBS overnight, and embedded in OCT. Cryostat sections (20 μm) were collected on Superfrost Plus slides (Fisher) and kept at −80°C. Slides were blocked in PHT (PBS, 1% heat-inactivated goat serum, 0.1% Triton X-100) for 1 hr at room temperature (RT), incubated overnight at 4°C with the primary antibody diluted in PHT, washed 3 times for 15 min at RT in PHT, incubated for 1 hr at RT with the fluorescently labeled secondary antibody diluted in PHT, washed 3 times for 15 min at RT in PHT and coverslip-mounted using Fluoromount G mounting media (Fisher).

Whole-mount immunohistochemistry of spinal cord explants was done as described above for tissue-section immunohistochemistry, except that 6 one hour washes were performed and that the fluorescently labeled secondary antibody was incubated overnight at 4°C. The TAG1 (clone 4D7, dilution 1:200) and neurofilament (clone 2H3, dilution 1:200) monoclonal antibodies were obtained from the Developmental Studies Hybridoma Bank (University of Iowa). The rabbit polyclonal GFP antibody (dilution 1:400) was from Molecular Probes. The rabbit polyclonal Rig-1, Robo1, and Robo2 antibodies (dilution 1:1000) were generated using Fc-tagged ectodomains as antigens.

In Situ Hybridization

In situ hybridization of rat spinal cords was carried out essentially as described in Fan and Tessier-Lavigne (1994). Fluorescent in situ hybridization of mouse spinal cords was carried out as described in the TSA plus protocol (Perkin Elmer).

Binding Experiments

Conditioned media from cells transfected with C-myc-hSlit2 was used in cell overlay assays to detect binding to COS cells transiently transfected with control, rRobo1, mRig-1, or rDCC expression vectors essentially as described in Keino-Masu et al. (1996).

Lipophilic Dye Tracing

Spinal cords of E12.5 *Rig-1* mutant and wild-type embryos were prepared in an open-book configuration, fixed with 4% paraformaldehyde, and injected with DiI (Molecular Probes) using iontophoresis into the dorsal region. DiI was allowed to diffuse for two days to label commissural axons along their entire length, enabling their visualization by conventional fluorescence microscopy.

Quantification

Unless otherwise state, a minimum of three embryos were observed for every genotype described and a minimum of five sections per embryo.

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References

Altman, J., and Bayer, S.A. (1984). The development of the rat spinal cord. Adv. Anat. Embryol. Cell Biol. *85*, 1–64.

Bovolenta, P., and Dodd, J. (1990). Guidance of commissural growth cones at the floor plate in embryonic rat spinal cord. Development *109*, 435–447.

Brose, K., and Tessier-Lavigne, M. (2000). Slit proteins: key regulators of axon guidance, axonal branching, and cell migration. Curr. Opin. Neurobiol. *10*, 95–102.

Brose, K., Bland, K.S., Wang, K.H., Arnott, D., Henzel, W., Goodman, C.S., Tessier-Lavigne, M., and Kidd, T. (1999). Slit proteins bind Robo receptors and have an evolutionarily conserved role in repulsive axon guidance. Cell *96*, 795–806.

Dodd, J., Morton, S.B., Karagogeos, D., Yamamoto, M., and Jessell, T.M. (1988). Spatial regulation of axonal glycoprotein expression on subsets of embryonic spinal neurons. Neuron *1*, 105–116.

Fan, C.-M., and Tessier-Lavigne, M. (1994). Patterning of mammalian somites by surface ectoderm and the notochord: Evidence for sclerotome induction by a Hedgehog homolog. Cell *79*, 1175–1186.

Fazeli, A., Dickinson, S.L., Hermiston, M.L., Tighe, R.V., Steen, R.G., Small, C.G., Stoeckli, E.T., Keino-Masu, K., Masu, M., Rayburn, H., and Simons, J. (1997). Phenotype of mice lacking functional Deleted in colorectal cancer (Dcc) gene. Nature *386*, 796–804.

Itoh, A., Miyabayashi, T., Ohno, M., and Sakano, S. (1998). Cloning and expressions of three mammalian homologues of *Drosophila* slit suggest possible roles for Slit in the formation and maintenance of the nervous system. Brain Res. Mol. Brain Res. *62*, 175–186.

Kaprielian, Z., Runko, E., and Imondi, R. (2001). Axon guidance at the midline choice point. Dev. Dyn. *221*, 154–181.

Keino-Masu, K., Masu, M., Hinck, L., Leonardo, E.D., Chan, S.S.-Y., Culotti, J.G., and Tessier-Lavigne, M. (1996). Deleted in colorectal cancer (DCC) encodes a netrin receptor. Cell *87*, 175–185.

Keleman, K., Rajagopalan, S., Cleppien, D., Teis, D., Paiha, K., Huber, L.A., Technau, G.M., and Dickson, B.J. (2002). Comm sorts robo to control axon guidance at the *Drosophila* midline. Cell *110*, 415–427.

Kidd, T., Brose, K., Mitchell, K.J., Fetter, R.D., Tessier-Lavigne, M.,

Goodman, C.S., and Tear, G. (1998a). Roundabout controls axon crossing of the CNS midline and defines a novel subfamily of evolutionarily conserved guidance receptors. Cell *92*, 205–215.

Kidd, T., Russell, C., Goodman, C.S., and Tear, G. (1998b). Dosage-sensitive and complementary functions of roundabout and commissureless control axon crossing of the CNS midline. Neuron *20*, 25–33.

Kidd, T., Bland, K.S., and Goodman, C.S. (1999). Slit is the midline repellent for the Robo receptor in *Drosophila*. Cell *96*, 785–794.

Li, H.S., Chen, J.H., Wu, W., Fagaly, T., Zhou, L., Yuan, W., Dupuis, S., Jiang, Z.H., Nash, W., Gick, C., et al. (1999). Vertebrate slit, a secreted ligand for the transmembrane protein roundabout, is a repellent for olfactory bulb axons. Cell *96*, 807–818.

Long, H., Sabatier, C., Ma, L., Plump, A.S., Yuan, W., Ornitz, D., Tamada, A., Murakami, F., Goodman, C.S., and Tessier-Lavigne, M. (2004). Conserved roles for Slit and Robo proteins in midline commissural axon guidance. Neuron *42*, in press.

Mombaerts, P., Wang, F., Dulac, C., Chao, S.K., Nemes, A., Mendelsohn, M., Edmondson, J., and Axel, R. (1996). Visualizing an olfactory sensory map. Cell *87*, 675–686.

Nguyen Ba-Charvet, K.T., Brose, K., Marillat, V., Kidd, T., Goodman, C.S., Tessier-Lavigne, M., Sotelo, C., and Chedotal, A. (1999). Slit-2 mediated chemorepulsion and collapse of developing forebrain axons. Neuron *22*, 463–473.

Plump, A.S., Erskine, L., Sabatier, C., Brose, K., Epstein, C.J., Goodman, C.S., Mason, C.A., and Tessier-Lavigne, M. (2002). Slit1 and Slit2 cooperate to prevent premature midline crossing of retinal axons in the mouse visual system. Neuron *33*, 219–232.

Rodriguez, I., Feinstein, P., and Mombaerts, P. (1999). Variable patterns of axonal projections of sensory neurons in the mouse vomeronasal system. Cell *97*, 199–208.

Serafini, T., Kennedy, T.E., Galko, M.J., Mirzayan, C., Jessell, T.M., and Tessier-Lavigne, M. (1994). The netrins define a family of axon outgrowth-promoting proteins homologous to *C. elegans* UNC-6. Cell *78*, 409–424.

Serafini, T., Colamarino, S.A., Leonardo, E.D., Wang, H., Beddington, R., Skarnes, W.C., and Tessier-Lavigne, M. (1996). Netrin-1 is required for commissural axon guidance in the developing vertebrate nervous system. Cell *67*, 1001–1014.

Shirasaki, R., Katsumata, R., and Murakami, R. (1998). Change in chemoattractant responsiveness of developing axons at an intermediate target. Science *279*, 105–107.

Stein, E., and Tessier-Lavigne, M. (2001). Hierarchical organization of guidance receptors: silencing of netrin attraction by slit through a Robo/DCC receptor complex. Science *291*, 1928–1938.

Tear, G., Harris, R., Sutaria, S., Kilomanski, K., Goodman, C.S., and Seeger, M.A. (1996). commissureless controls growth cone guidance across the CNS midline in *Drosophila* and encodes a novel membrane protein. Neuron *16*, 501–514.

Tessier-Lavigne, M., and Goodman, C.S. (1996). The molecular biology of axon guidance. Science *274*, 1123–1133.

Tessier-Lavigne, M., Placzek, M., Lumsden, A.G.S., Dodd, J., and Jessell, T.M. (1988). Chemotropic guidance of developing axons in the mammalian central nervous system. Nature *336*, 775–778.

Yu, T.W., and Bargmann, C.I. (2001). Dynamic regulation of axon guidance. Nat. Neurosci. *4*, 1169–1176.

Yuan, S.-S.F., Cox, L.A., Dasika, G.K., and Lee, E.Y.-H.P. (1999). Cloning and functional studies of a novel gene aberrantly expressed in RB-deficient embryos. Dev. Biol. *207*, 62–75.

Zou, Y., Stoeckli, E., Chen, H., and Tessier-Lavigne, M. (2000). Squeezing axons out of the gray matter: A role for Slit and Semaphorin proteins from midline and ventral spinal cord. Cell *102*, 363–375.